6

Pigments of Myxobacteria

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Colors in nature arise either through absorption by certain molecules or atoms of selected wavelengths in the visible range, or through interference of light waves in material with a special physical structure. Structural colors are observed with some cytophagas: Their delicate swarm colonies may act like a thin film and show iridescence (Reichenbach and Dworkin 1981). The color of myxobacteria seems to be due exclusively to pigments with various chemical chromophores. The pigments are either localized within the cell or they are excreted. The extracellular pigments may be excreted as such, or they may arise in the environment through the action of enzymes liberated from the cells.

The myxobacteria are indeed a colorful assembly of organisms. The cell mass of most strains is more or less intensely yellow, orange, red, or purple. The fruiting bodies are almost always colored, and often very brightly so, in hues of yellow, orange, red, purple, light-to-dark brown, and even black, although rarely. The only myxobacterium that is often totally unpigmented is *Myxococcus stipitatus*. This organism, however, produces a substance of unknown chemical composition with a strong yellow fluorescence (Lampky and Brockman 1977) that seems to be excreted. It should be mentioned that there are some *M. stipitatus* strains with fawn-to-red brown swarms and fruiting bodies.

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Chemical Structures and Biosynthesis

Intracellular Pigments

It was suggested, although on quite insufficient evidence (Jahn 1924; Reichenbach 1965b; Burchard and Dworkin, 1966a), that the color of the cells of the myxobacteria is due to carotenoids. After some very preliminary attempts to investigate this assumption (Greene and Leadbetter 1962; Renwick and Peterson 1969), it was indeed corroborated. The intracellular pigments of a variety of myxobacteria were extracted, separated into pure compounds, and characterized by chemical and physical methods (Kleinig and Reichenbach 1969, 1973b; Kleinig *et al.* 1970, 1971; Reichenbach and Kleinig 1971, 1972). From these studies the following picture emerges.

Myxobacteria always contain mixtures of different carotenoids. Up to 60 different compounds have been obtained from one single strain, most of them, however, in minute quantities. It is thus obvious that spectra of crude pigment extracts cannot be very useful by themselves to characterize an organism, particularly as the quantitative relationships between the different pigments depend on the culture conditions, as well as on the specific strain. The myxobacterial carotenoids are of normal length (C_{40}) and either acyclic or monocyclic (Fig. 6.1). They show increasing degrees of desaturation, almost up to the theoretical endpoint (torulene, dehydrolycopene, aromatic carotenoids). Many contain hydroxyl and keto functions in various positions and combinations. In the hydrocarbon series, the complete Porter–Lincoln series (phytoene, phytofluene, ζ-carotene, neurosporene) can usually be demonstrated. The hydroxyl groups may be substituted with a sugar molecule, usually glucose, but rhamnose has also been found (Figs. 6.2, 6.3). Although unknown in eukaryotic organisms, carotenoid glycosides are not uncommon in bacteria (Isler 1971; Liaaen-Jensen and Andrewes 1972; Liaaen-Jensen 1973). In myxobacteria, as in most other bacteria, the sugar is attached to a tertiary carbon atom (tertiary glycosides: usually at a 1′-hydroxyl). The cyanobacteria, in contrast, produce secondary (2′-hydroxyl) glycosides. All myxobacterial glucosides, but not the rhamnosides, have been found to be esterified with a fatty acid at one of the hydroxyls of the sugar moiety. Although this fatty acid is variable, the fatty acid pattern of the carotenoid glucosides does not simply

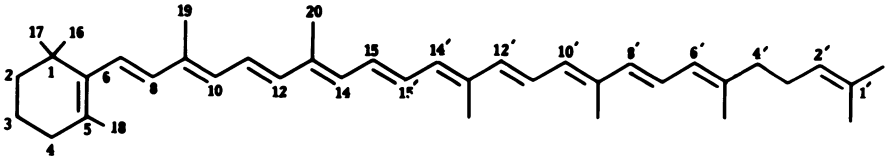

Figure 6.1. β,ψ-Carotene, the chromophore of many myxobacterial carotenoid pigments, showing the numbering of the carbon atoms.

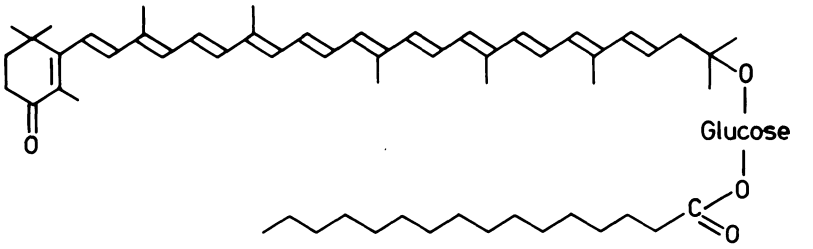

Figure 6.2. Myxobacton palmitate.

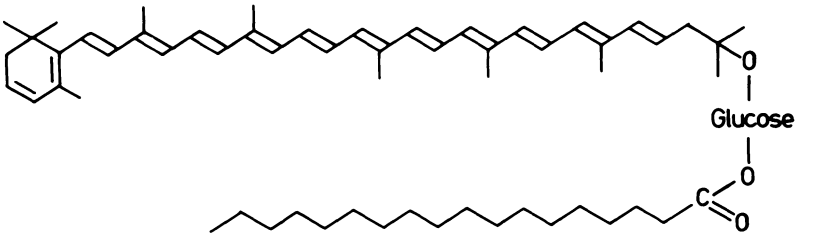

Figure 6.3. Myxobactin stearate.

reflect the overall fatty acid pattern of the cell: The latter is dominated by branched fatty acids, whereas the pigment glucosides contain mainly straight-chain fatty acids. Carotenoid glucoside fatty acid esters have been described in only one other organism: *Nocardia kirovani* (Vacheron *et al.* 1970). It is, however, questionable whether they are really so rare. In many laboratories, the crude pigment extracts are saponified before being chromatographed, in which case such esters would be hydrolyzed. *Myxococcus fulvus* produces another remarkable carotenoid derivative: a direct 2′-hydroxy fatty acid ester. In this case, too, the hydroxyl is esterified with a variety of straight-chain fatty acids. Although carotenoid fatty acid esters are quite common in higher plants, they have not been described previously in bacteria. However, again the possibility of hydrolysis during isolation has to be considered. Truly unique pigments are produced by *Nannocystis exedens*. Here, the main pigments are moncyclic aromatic carotenoids with highly substituted benzene rings (1,2,5- and 1,2,4,5-methylated and an additional hydroxyl group in 3-position; Fig. 6.4). Some of these pigments have a conjugated keto group in 2′-position, which shifts the electronic absorption spectrum to exceptionally long wavelengths (λ max 493 nm, ethanol) rarely reached by any carotenoid; these pigments have a deep blue–purple color (Kleinig *et al.*, to be published). Another unusual chromophore is that of myxobactin (see Fig. 6.3). This pigment is a monocyclic carotenoid glucoside with a 3,4-unsaturated β-carotene ring not known in any other natural pigment.

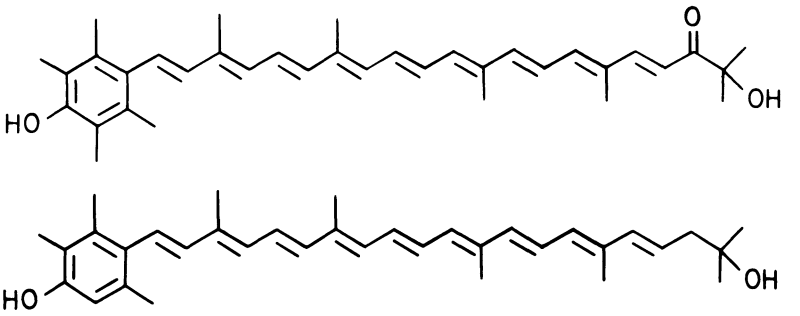

Figure 6.4. The main pigments of *Nannocystis exedens.*

Quantitatively, the main pigments of myxobacteria are almost always carotenoid glycosides, usually glucosides. Rhamnosides have so far been found only in *Sorangium compositum.* The chromophores of the glycosides differ characteristically in the two suborders of the *Myxobacterales* (see Chapter 1). In the *Cystobacterineae,* we find monocyclic carotenoids with a 4-keto (myxobacton) or a 3,4-unsaturated (myxobactin) β-carotene ring, and generally a dominance of dark-red-to-purple keto pigments. In the *Sorangineae,* the chromophores are acyclic or monocyclic, with an unsubstituted or a 3-hydroxylated β-carotene ring, and keto pigments are completely lacking. Consequently, the color of the cell mass is more in the red–orange range in the former case and yellow–orange in the latter. The phenotypic appearance of an organism depends on the carotenoid pattern, the quantitative relations of the individual pigments, and the occurrence of additional noncarotenoid pigments. Thus, *M. fulvus* and *Myxococcus xanthus* seem to have an identical carotenoid pattern, but in *M. xanthus* the dominating pigments absorb at shorter wavelengths. *Myxococcus xanthus* and *Myxococcus virescens* seem to contain the same carotenoids, but *M. virescens* in addition produces appreciable quantities of a greenish-yellow pigment of unknown structure that is also excreted into the environment.

Nannocystis exedens is exceptional among the myxobacteria in that it does not contain any carotenoid glycosides but contains instead the peculiar monocyclic aromatic carotenoids mentioned above, which are not found in any other myxobacterium.

As summarized in Table 6.1, the larger taxonomic groups of myxobacteria are characterized by different pigment types. Thus, the pigments are to a certain degree useful as chemosystematic markers. In this connection it is also interesting to compare the myxobacteria with other unicellular gliding bacteria. Carotenoids have been described in *Cytophaga* and *Flexibacter* strains, mainly the bicyclic C_{40} carotenoids zeaxanthin (3,3′-dihydroxy-β,β-carotene) and its biosynthetic precursors, cryptoxanthin (3-hydroxy-β,β-carotene) and β,β-carotene. With many *Cytophaga*-like bacteria, including *Sporocytophaga,* the dominant pigments are, however, of the flexirubin type (Fig. 6.5), a novel

Table 6.1. The Occurrence of Various Types of Intracellular Pigments in Different Myxobacteria and *Cytophaga*-Like Bacteria

Organism	Carotenoids[a]								Flexirubin-type pigments
						Glucosides			
	Di-	Mono-	Acyclic	Aromatic	With keto functions	Chromophore with keto functions	Chromophore with hydroxyl functions	Rhamnosides	
Myxobacterales									
Cystobacterineae									
Myxococcus fulvus	−	+	+	−	+	+	−	−	−
M. xanthus	−	+	+	−	+	+	−	−	−
M. virescens	−	+	+	−	+	+	−	−	−
Cystobacter fuscus	−	+	+	−	+	+	−	−	−
C. ferrugineus	−	+	+	−	+	+	−	−	−
C. velatus	−	+	+	−	+	+	−	−	−
Stigmatella aurantiaca	−	+	+	−	+	+	−	−	−
Myxobacterales									
Sorangineae									
Chondromyces apiculatus	−	+	+	−	−	−	+	−	−
Sorangium compositum	−	+	+	−	−	−	+	+	−
S. cellulosum	−	+	+	−	−	−	+	−	−
Nannocystis exedens	−	+	+	+	+	−	−	−	−
Cytophaga-like bacteria									
Cytophaga spp.	+	−	(+)	−	−				+
Flexibacter spp.	+	−	(+)	−	−				+
Sporocytophaga sp.									+

[a]Symbols: +, occurrence demonstrated; (+) traces; −, excluded; open, not known.

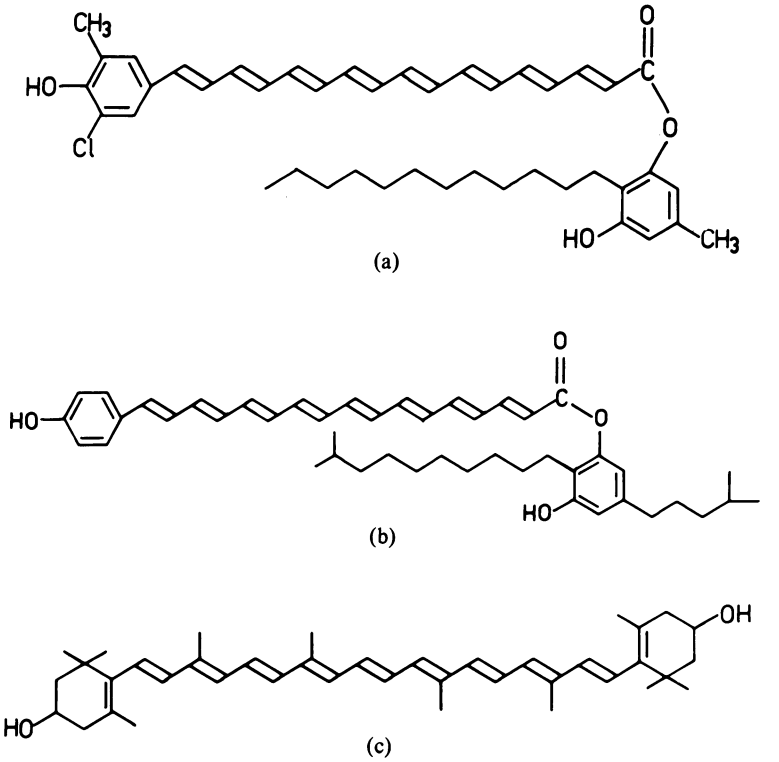

Figure 6.5. Typical pigments of *Cytophaga*-like bacteria: (a) Chloroflexirubin from *Flexibacter elegans;* (b) flexirubin-type pigments from *Cytophaga johnsonae:* (c) 3,3′-dihydroxy-β,β-carotene or zeaxanthin.

class of pigments discovered in *Flexibacter elegans* and so far found only in these bacteria and in certain flavobacteria (Achenbach *et al.* 1978; Reichenbach *et al.* 1981). Little is known about the pigments of *Lysobacter.* These organisms seem to produce mainly or exclusively extracellular pigments.

In addition to carotenoids other intracellular pigments may be found in certain strains of myxobacteria. *Myxococcus xanthus* FB produces a yellow non-carotenoid pigment with an absorption maximum at 379 nm that accumulates in stationary phase cells and becomes particularly conspicuous in cultures grown in the dark when carotenoid synthesis is much reduced. The same organism also contains protoporphyrin IX, although this pigment becomes visible only after extraction (Burchard and Dworkin 1966a). The pale yellow antibiotic myxalamid is synthesized by strains of several myxobacteria (*M. xanthus, M. virescens, M. stipitatus,* and *Stigmatella aurantiaca*) in sufficient quantity to become visible in the culture. Although it can be extracted from the cell mass, it is not absolutely certain that it is really intracellular (Gerth *et al.* 1983). Other intracellular antibiotics with a chromophore, such as yellow

myxovirescin (Gerth *et al.* 1982), are present, as a rule, in minute amounts and thus do not ordinarily contribute to the color of the culture.

Extracellular Pigments

Little is known about the chemical nature of the extracellular pigments of myxobacteria. *Myxococcus virescens* excretes a characteristic greenish-yellow water-soluble pigment into the medium. While this pigment seems to be excreted in its finished form, this is not the case with the dark brown or black pigments appearing in cultures of several myxobacteria (some *M. fulvus, Cystobacter* and *Stigmatella* strains). For example, cultures of *Stigmatella aurantiaca* in peptone liquid medium may become pitch black within 1 or 2 hr after having reached stationary phase (Reichenbach and Dworkin 1969). Very probably all these pigments are melaninlike and arise through the action of phenol oxidases that are either excreted or set free when the cells lyse.

Certain *Cystobacter* strains, either physiologic variants of *Cystobacter velatus* or a species of its own (*Cystobacter violaceus:* Kühlwein and Gallwitz 1958), excrete a deep violet pigment into the medium. The pigment is mainly produced in old and degenerating cultures on rich media in presence of oxygen. Its physiocochemical properties suggest that it is a melaninlike material, although the relevant enzymes have not been demonstrated (Reichenbach 1965b).

The specific fruiting body pigments also have to be regarded as extracellular. They are located within the sporangiole walls, in the slime matrix, and in the slime envelopes. They often are very bright and intense in color and seem to be very resistant to degradation. Nothing is known about their chemistry. The dark and red–brown pigments are perhaps also melanins. The fruiting body pigments are formed relatively late during morphogenesis (see Chapter 1, Fig. 1.10a).

Biosynthesis

It appears that carotenoids are synthesized by myxobacteria from mevalonic acid via "lycopersene" and phytoene in the classic way by a series of symmetrical dehydrogenation steps with or without later cyclization of one end to form a β-carotene ring. It is not known why in all myxobacteria ring closure occurs on only one end. The biosynthetic scheme is supported by efficient incorporation of radioactive mevalonic acid into myxobacterial carotenoids (Kleinig and Reichenbach 1973a) and by the regular occurrence of the Porter–Lincoln series in myxobacteria. For a better understanding of the reaction sequence beyond the level of phytoene, studies with certain metabolic inhibitors were undertaken (Kleinig and Reichenbach 1973a; Kleinig 1974, 1975). In *M. fulvus,* D- or L-nicotine at concentrations below 1 m*M* specifically inhibited cycli-

zation of the newly synthesized carotenoids. Thus, instead of the usual monocyclic myxobacton (Fig. 6.2) a new, acyclic carotenoid glucoside was formed. When the nicotine was then removed and the culture incubated anaerobically, the acyclic glucoside was transformed into another new glucoside with a 3′,4′-didehydro-β,ψ-carotene chromphore, that is, cyclization but not oxydation took place. If the culture was then aerated, the keto function at the 4-position of the β-carotene ring was introduced to give myxobacton. Incubation under aerobic conditions, after removal of nicotine, led directly to myxobacton. In presence of higher concentrations of nicotine (1–4 m*M*), a second effect was observed: Introduction of the tertiary hydroxyl in 1′-position was blocked, so that glucosides could not be synthesized any more, and finally over 95% of the total pigment was lycopene. During all these experiments, the specific pigment content of the cultures (i.e., amount of pigment per cell) remained constant (about 0.42 nmol/mg dry cell mass, culture under blue light, 2500 lux). The same effects as with nicotine were also observed with the inhibitor 2-(4-chlorophenylthio) triethylamine (CPTA) hydrochloride.

The lycopene that accumulated in presence of the inhibitors was not used for the synthesis of other carotenoids when the inhibitor was removed. Indeed, lycopene, which is a highly oxydized structure, is not a likely precursor for the cyclization step or other enzymatic reactions. However, if after removal of nicotine another inhibitor, the herbicide Sandoz (San) 6706, was added, at least some of the lycopene was reintroduced into the biosynthetic pathway and converted into myxobacton. San 6706, 4-chloro-5-(dimethylamino)-2-α,α,α (trifluoro-m-tolyl)-3(2H)-pyridazinone, specifically prevents the desaturation of phytoene to the colored carotenoids, as does diphenylamine (DPA). While DPA is incorporated into the cytoplasmic membrane and cannot quantitatively be removed again, San 6706 has the advantage that it can easily be washed out.

The DPA at very low concentrations (0.001–0.01 m*M*) led to a considerable increase in the specific pigment content that was due, however, almost entirely to phytoene. A new acyclic 7,8-desaturated carotenoid glucoside also appeared (6% of the total pigment at a DPA concentration of 0.002 m*M*). When phytoene desaturation was blocked with San 6706, phytoene also accumulated, but in this case the specific pigment content remained constant. As soon as the inhibitor was removed, a fast synthesis of myxobacton and 4-keto-torulene, the main carotenoids of the strain, began, and the concentrations of the two pigments rose steeply to normal levels. Curiously, the phytoene pool remained high, although some conversion of radioactive phytoene into colored carotenoids was demonstrated. Obviously, phytoene synthesis remained high initially to supply the urgently needed precursor molecules; there was, however, only limited exchange between the molecule populations, and the biosynthetic pathway leading to the colored compounds was preferentially fed with newly synthesized phytoene. Experiments with chloramphenicol showed that this efficient regulation of carotenoid biosynthesis took place with preexisting enzymes.

From these studies we may cautiously deduce that in *M. fulvus,* as in other organisms, a sequence of closely linked enzyme reactions is involved in carotenoid biosynthesis (phytoene to myxobacton or to lycopene) but that this sequence is not so rigid that use of unusual precursors (lycopene, acyclic glucosides) is excluded. It is not yet clear at what stage the glycosylation step normally takes place. It may happen relatively early in pigment synthesis, as is suggested by the occurrence of partly desaturated acyclic carotenoid glycosides. In any case, carotenoid glycosides are still available for biochemical modification of the chromophore (cyclization, introduction of keto function). There may indeed be a certain flexibility in the pathway.

The inhibitor experiments allow certain conclusions to be drawn with respect to carotenoid turnover. The absolute concentrations of the highly desaturated pigments, myxobacton and 4-keto-torulene, which were no longer synthesized in inhibited but growing cultures of *M. fulvus,* remained completely constant over 22 hr or four generations, even under (mild) illumination of the bacteria (2500 lux, blue light). As mentioned above, accumulated lycopene also remained unchanged under normal culture conditions, and when the inhibitor San 6706 was added it was only transformed into other pigments and was not decomposed. Thus, carotenoids once synthesized are not easily catabolized.

Physiology

The carotenoids of myxobacteria are localized in the cytoplasmic membrane of the cell (Kleinig 1972). Their synthesis is stimulated in many cases, but not always, by light; in illuminated cultures, the carotenoid concentration may rise by a factor of 10–20 (Reichenbach 1965b; Burchard and Dworkin 1966a; Grimm 1967; Reichenbach 1971; Kleinig and Reichenbach 1973b). The mechanism of this induction is not clear. It is sufficient to illuminate the cultures for a short time and then to incubate them in the dark, even at low temperature (6°C), to elicit the full response. In *M. xanthus* FB, the action spectrum of induction shows maxima at 410 nm and, to a lesser degree, at 512, 533, 548, 585, and 635 nm (Burchard and Hendricks 1969). As these maxima correspond to the absorption maxima of a porphyrin and protoporphyrin IX is known to be a photosensitizing pigment in FB (Burchard and Dworkin 1966), it was proposed that protoporphyrin IX may be responsible for carotenoid induction.

In growing cultures, the specific carotenoid content is constant and tightly regulated (Kleinig 1975) albeit, of course, dependent on environmental factors such as light. Carotenoid content increases, however, in late logarithmic and stationary phase (Burchard and Dworkin 1966a); obviously, the machinery for pigment synthesis remains fully active, or nearly so, for some time after growth. In *M. fulvus* Mx f2, the carotenoid content of acetone extracted dry cells was 0.003% when grown exponentially in the dark, 0.03% when grown

exponentially in the light, and 0.06% when grown in late log phase (Reichenbach and Kleinig 1971). The carotenoid content also depends on the strain. By treatment with the mutagen *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine, it was possible to obtain mutant strains from Mx f2 with either decreased or increased pigment content, although at the same time the pigment pattern, too, was changed.

At least one of the functions of the carotenoids in myxobacteria has been established. In *M. xanthus* FB, the carotenoids prevent photolysis, which is mediated by a sensitizer, protoporphyrin IX, produced by the same organism (Burchard and Dworkin 1966a; Burchard *et al.* 1966). Only when the cells are grown in the light, however, do they contain sufficient carotenoid to be resistant to photolysis; when they are grown in the dark, the carotenoid levels become so low that they are no longer protective, and the cells quickly lyse when exposed to light in the stationary phase. Photolysis is also observed when cultures are grown in the light in presence of 10^{-4} *M* diphenylamine, which prevents the synthesis of colored carotenoids. On the other hand, mutants that efficiently synthesize carotenoids in the dark do not become sensitive to light when grown in the dark.

In *M. fulvus* Mx f2, cultures can be obtained that have a low content of colored carotenoids or are completely carotenoid free, either by using unpigmented mutants or by growing the wild strain in presence of inhibitors such as diphenylamine or the herbicide, San 6706 (see above). Under aerobic conditions such cultures become very sensitive to illumination with daylight intensities (about 40,000 lux); the cells are killed within minutes. Wild type cells, in contrast, survive such intense illumination for a long time (Reichenbach, unpublished data). Thus, there is no question that the carotenoids have a photoprotective effect in myxobacteria, as is known from other bacteria, too.

The peculiar carotenoid–glucoside–fatty acid esters with their polar head and hydrophobic tails obviously fit particularly well into the membrane and might serve as structural elements and lipase-resistant stabilizers for the membrane in addition to performing their photoprotective function.

Pigmentation in myxobacteria is not always a stable character. When *M. xanthus* strain FB is plated, two types of colonies appear: one with a *tan* and another with a *yellow* color (Burchard and Dworkin 1966a). The frequency of the *yellow* type varies between 5 and 20%. Although most *yellow* colonies give *yellow* subclones only, the *tan* colonies usually shed a certain proportion of *yellow* clones upon subcultivation; stable *tan* strains can be obtained, however. It appears that both variants contain an identical set of carotenoid pigments but that the *yellow* variant produces in addition a noncarotenoid pigment, P 379, which in methanol has an absorption maximum at 379 nm. This pigment seems to be identical with the greenish-yellow pigment produced by *M. virescens* (Burchard *et al.* 1977b). In fact, the organisms classified as *M. virescens* may be merely stable *yellow* variants of *M. xanthus*. On the other hand, the appearance of the *tan* phenotype in *M. virescens* strains is also not unusual. Incidently, the expression of the *yellow* phenotype is sensitive to temperature:

At 36.5 °C, the temperature maximum for growth of FB, P 379 is not synthesized any longer (Burchard *et al.* 1977b). The proportion between *tan* and *yellow* cells can be influenced by a number of environmental factors (Sudo and Dworkin 1969; Burchard *et al.* 1977). An increase in the proportion of the *yellow* type is observed in cultures with a low cell density (below 10^6/ml). Ultraviolet (UV) irradiation leads to an increase of the *yellow* type among the survivors. The reason may be a higher resistance of *yellow* cells to UV damage. The UV inactivation curve of *yellow* cultures shows a shoulder not observed in the inactivation curve of *tan* cells. This seems to indicate a multiple-hit inactivation in the case of the *yellow* type. However, a stimulation of the conversion from *tan* to *yellow* by UV is also not excluded. An increase of the *yellow* type is further observed upon addition of mitomycin C, nalidixic acid, or phenethyl alcohol to the culture but not with acridine orange or ethidium bromide. A mechanistic explanation of the phenomenon is not yet available. Perhaps a genetic switch is at work, as is known for the phase variations of other bacteria. When the switch is thrown and the cells grow on plates, the system obviously remains stable for several generations, perhaps under the influence of the high cell density in colonies, for only rarely are colonies with an intermediate color or with color sectors seen.

In *Archangium (Cystobacter) violaceum* production of the characteristic deep-violet pigment was found to be variable and connected with a certain colony type (Reichenbach 1965b; Grimm 1967; Grimm and Kühlwein 1973a,b). Again, the conversion between slow spreading, soft slimy, intensely colored K colonies and fast spreading, tough slimy, poorly pigmented G or S colonies is controlled by environmental factors. Each colony type may become more or less stable under certain conditions, and variable under others. Thus, in liquid culture the conversion goes exclusively S to K, whereas on plates it goes into the opposite direction. The mechanism underlying this variability is not known.